Thioflavones as novel neuroprotective compounds

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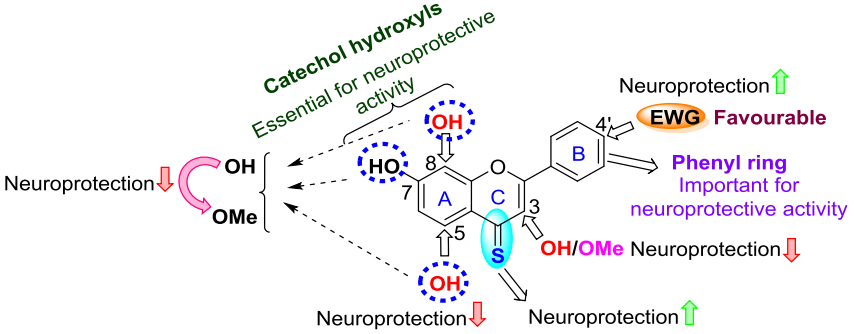
ABSTRACT: Increased levels of oxidative stress are linked with many neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, and Amyotrophic Lateral Sclerosis. Therapeutic strategies aimed at identifying small molecules that are capable of conferring neuroprotection against oxidative stress are therefore of significant importance for developing effective treatments for these neurodegenerative diseases. A library of 76 compounds containing hydroxy flavones, methoxy flavones and their 4-thio analogues has been evaluated for neuroprotection against H_2O_2-induced oxidative stress. This study revealed the novel 7,8-dihydroxy 4-thioflavones as neuroprotective compounds, with compounds **14f** and **18f** showing highest neuroprotective effects at lower concentrations (0.3 µM). The neuroprotective activities were found to be mediated *via* activation of the anti-apoptotic cell survival proteins of the ERK1/2 and PI3K/Akt pathways. Structure activity relationship analysis revealed that the presence of the B-ring phenyl group is essential for the greater neuroprotective activities of the flavones studied herein. Additionally, replacing the 4-C=O moiety with a 4-C=S moiety enhances neuroprotection. These findings form the basis for further optimisation and in-depth drug development for treating various neurological disorders.

Keywords: Flavones, neuroprotective, structure-activity relationship studies.

GRAPHICAL ABSTRACT



INTRODUCTION

Flavones, a subclass of flavonoids, are polyphenolic phytochemicals that have been well recognised for their diverse pharmacological activities such as antioxidant [1,2], anti-inflammatory [3,4], antimicrobial [5–7], cardioprotective [8,9], neuroprotective [10,11] and anticancer [12,13] activities. Due to their broad spectrum of pharmacological activities, the natural flavones have gained attention as potential medicinal chemistry scaffolds. In particular, several epidemiological *in vitro* and *in vivo* studies have highlighted the potential of flavonoids as neuroprotective agents. For example, natural flavonoids such as fisetin [14], luteolin, quercetin, myricetin and hesperetin [15–17] have been reported to protect neurons against oxidative damage. Further, several synthetic flavones and thioflavones [18] have limited neurodegeneration associated with a variety of neurological disorders, namely Alzheimer's disease [19] and Parkinson's disease [20]. Studies focusing on the blood–brain barrier (BBB)

permeability of flavonoids have highlighted that the lipophilic polymethoxy flavonoids possess greater BBB permeability than the polar flavonoids; the permeability potency of the compounds also correlated with their lipophilicity (log P) [21,22]. With our interest in developing novel flavone derivatives as therapeutic agents, we had previously synthesised and characterised a library of 76 hydroxy flavones, methoxy flavones and their 4-thio derivatives (Figure-1). As a result of the promising BBB permeability of methoxy flavones, as well as the absence of a systematic investigation of the effect of substitution of the 4-carbonyl (4-C=O) group by a 4-thiocarbonyl (4-C=S) group on the neuroprotective activities of flavones, we devised a programme of work to explore the structure-activity relationships (SARs) of the library of 76 flavones presented herein. Thus hydroxy and methoxy flavones, along with their 4-thio derivatives, were evaluated for their neuroprotective effects against H_2O_2-induced oxidative stressed SH-SY5Y neuroblastoma cell line. Further, the molecular mechanisms of action of the most potent neuroprotective flavones were investigated. On the basis of these results, SARs were deduced and the key structural features required for the neuroprotective activities were deciphered.

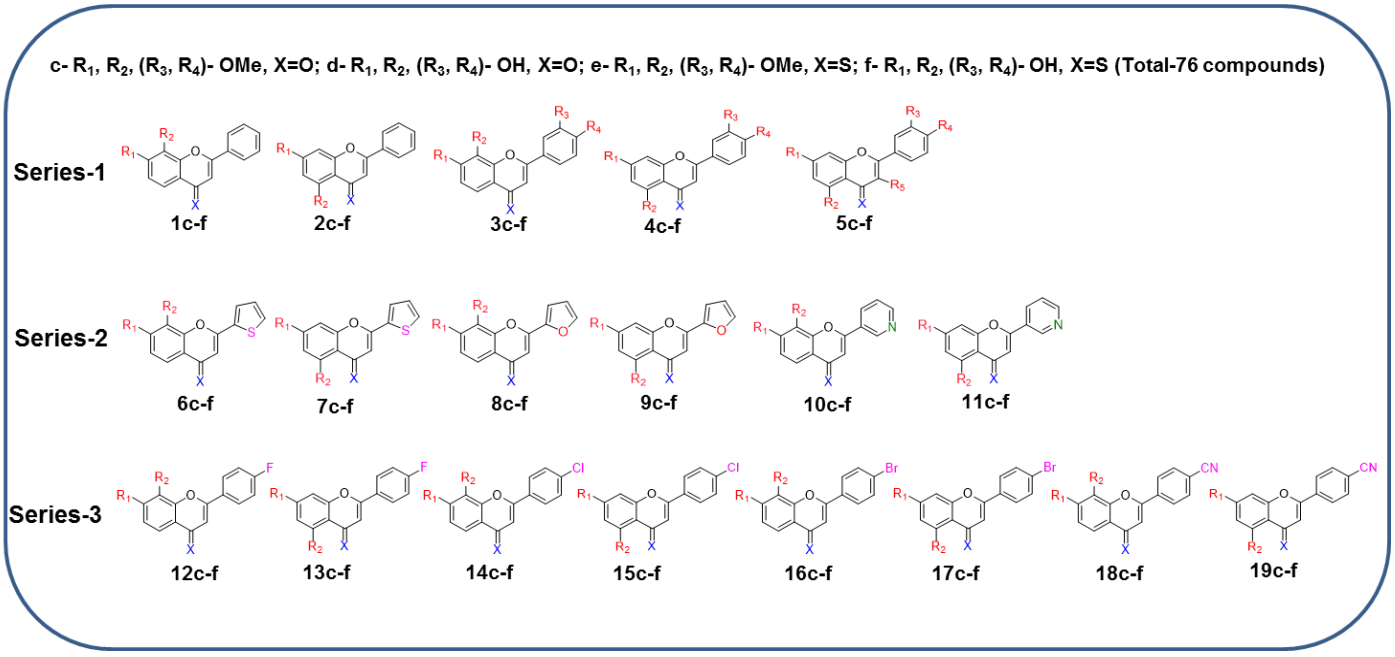


Figure 1. Structures of flavones mentioned in this study.

MATERIAL AND METHODS

Chemicals: DPPH, Ascorbic acid, EDTA, ferrous chloride hexahydrate, ferrozine, hydrogen peroxide and sterile PBS with Ca^{2+} and Mg^{2+} were purchased from Sigma-Aldrich, UK.

Cell line and Culture: SH-SY5Y (human neuroblastoma cell line) was purchased as a frozen stock from the European Collection of Cell Cultures (ECACC). SH-SY5Y cells were grown in DMEM:F-12 (1:1) [with sodium pyruvate] supplemented with 15% FBS and 1% NEAA in T-75 culture flasks (Greiner bio-one). All of the cell culture reagents were obtained from Lonza, UK. PathScan Intracellular Signaling array kit was purchased from Cell Signaling (Catalog number-7323). For the cell culture experiments, the stock solutions of the test compounds (10 mg/mL) were prepared in sterile DMSO and ethanol (1:1 v/v) and these stocks were then appropriately diluted with the complete culture medium and the ethanol and DMSO levels were maintained below 1% in the test concentrations. The IC_{50} values were calculated using Graphpad prism 6.

H_2O_2-IC_{50} determination: SH-SY5Y cells were seeded at a density of 1.5 x 10^5 cells/mL into 96 well plates and incubated for 72 h. After 72 h, the medium was aspirated and the cells were washed twice with sterile PBS and then treated with a range of concentrations (0 to 1000 µM) of H_2O_2 in sterile PBS (with Ca^{2+} and Mg^{2+}) for 1 h. After 1 h, H_2O_2 solution in the well was replaced with the warm medium and incubated for 17 h. After the 18 h exposure, the cell viability was determined using the MTT

assay[23]. The MTT assay involved the addition of 20 µL of MTT (5mg/mL) solution in PBS into each well followed by the incubation of the cells for 5 h. The purple formazan crystals formed were dissolved in 100 µL of DMSO and the plates were read at 570 nm using a SPECTRA max UV spectrometer (Bio-Rad). The data represented are the mean of the three individual experiments. The cell viability of the control is considered to be 100%.

Neuroprotective assay: SH-SY5Y cells were seeded at a density of 1.5 x 10^5 cells/mL into 96 well plates and incubated for 48 h. After 48 h, the cells were treated with these synthesised derivatives at a single dose of 3 µM or at a range of concentrations (0, 0.1, 1 and 3 µM) for 24 h. After 24 h of this treatment with the synthesised compounds, the medium was aspirated and the cells were washed twice with sterile PBS and then treated with 130 µM of H_2O_2 (IC_{50} concentration) in sterile PBS (with Ca^{2+} and Mg^{2+}) for 1 h. After 1 h, the H_2O_2 solution in the well was replaced with the warm medium and incubated for 17 h. After the 18 h exposure, the cell viability was determined using the MTT assay (as described earlier in the H_2O_2-IC_{50} determination section). The data represented are the mean of the three individual experiments. The cell viability of the control is considered to be 100%.

Neurotoxicity assay: SH-SY5Y cells were seeded at a density of 1.5 x 10^5 cells/mL into 96 well plates and incubated for 48 h. After 48 h, the cells well treated with these synthesised derivatives at 3 µM and 10 µM concentrations for 24 h. Following the 24 h exposure, the cell viability was assessed using MTT assay (as described previously). The cell viability of the control is considered to be 100%.

DPPH Radical Scavenging Assay: The free radical scavenging activities of the samples were measured using the stable DPPH radical. The free radical scavenging activity of the samples was analyzed according to the method of Sulaiman *et al* [24]. Briefly, 0.3 mM solution of DPPH in ethanol was prepared. Test compounds (50 µL) (concentration-3 µM in ethanol) were added to 150 µL of the DPPH

solution in each well of a 96-well plate. For the blank, only 50 µL of ethanol was added to the DPPH solution. The plates were incubated in the dark for 30 min at 37 °C. After 30 min, the decrease in absorbance was measured at 515 nm using a microplate reader (Spectramax 340PC). All tests were performed in triplicate. The capability to scavenge the DPPH radicals was calculated using the equation: (%) = [(Absorbance of control – Absorbance of sample)/ Absorbance of control] × 100. Ascorbic acid was used as a positive control. This protocol was validated by the determination of the IC_{50} of ascorbic acid (concentration range tested 1000 µM – 1 µM) and the IC_{50} value was found to be 330 $\pm$ 0.5 µM (Supplementary info, Figure-S2). All experiments were performed in triplicate.

Metal chelating assay: The chelating activity for ferrous ions was assessed according to the method of Dinis *et al* [25]. 50 µL of test compounds (concentration-3 µM in ethanol), and deionized water (130 µL) were added to 96 well plates. 5 µL of ferrous chloride hexahydrate (2 mM) was added to each well and incubated for 5 min. 15 µL of ferrozine (5 mM) was added to initiate the ferrous-ferrozine complex reaction. After 10 min of incubation at room temp, the absorbance was measured at 562 nm using a microplate reader (Spectramax 340PC). The metal chelating ability of the test compounds was determined using the equation: (%) = [(Absorbance of control – Absorbance of sample)/ Absorbance of control] × 100. EDTA was used as a positive control. This protocol was validated by the determination of the IC_{50} of EDTA (concentration range tested 1000 µM – 1 µM) and the IC_{50} value was found to be 42 $\pm$ 0.8 µM (Supplementary info, Figure-S3). All experiments were performed in triplicate.

PathScan sandwich immunoassay: The PathScan Intracellular Signalling array kit was used for the simultaneous detection of 18 significant and well-characterised cellular proteins and signalling nodes that were phosphorylated or cleaved at the specific residues.

Preparation of cell lysate: SH-SY5Y cells (2.5 x 10^5 cell/mL) were seeded into 24 well plates (2 mL/well) and incubated for 48 h. After 48 h, the cells were treated with the test compounds at 3 µM concentration for 24 h. After 24 h of this treatment, the medium was aspirated and the cells were washed twice with sterile PBS and then exposed to 130 µM of H_2O_2 (IC_{50} concentration) in sterile PBS (with Ca^{2+} and Mg^{2+}) for 1 h. Following this H_2O_2 insult, the cells were washed with ice-cold 1X phosphate-buffered saline and lysed in 1X cell lysis buffer provided (phosphotase and protease inhibitors added). These lysates were quantified using the BCA protein assay.

Assay procedure: The array blocking buffer was added to each well of the glass slide provided and incubated for 15 min at room temperature. Subsequently, the cell lysate, diluted to 0.3 mg/mL in array diluents, was added to each well and incubated for 2 h at room temperature. Subsequent to washing, the detection antibody cocktail was added to each well and incubated for 1 h at room temperature. Horseradish peroxidase (HRP)-linked streptavidin was added to each well and incubated for 30 min at room temperature. The slide was then covered with LumiGLO/Peroxide reagent (Cell Signaling Technology) and exposed to film for 2–30 sec. The image was captured by a digital imaging system, ImageQuant LAS 4000 (GE Healthcare).

BBB score prediction: BBB scores presented in this study were determined using the online, free access Blood-Brain Barrier Prediction Server- CBLigand, available from http://www.cbligand.org/BBB/ [Accessed 5th February 2016]. SVM algorithm and MACCS fingerprint were selected for the calculation. The predicted BBB scores for all 76 flavones presented in this study are provided in Table-S1, Supplementary Information.

RESULTS AND DISCUSSION

Neuroprotective evaluation

The flavone scaffolds presented in this study were designed according to medicinal chemistry strategies such as the bioisostere approach and Topliss scheme, and this resulted in three series of flavones; series-1 contained well-known flavones with different number of hydroxyls and their derivatives, series-2 contained bioisoteric analogues of the active flavone (determined from series-1) and series-3 contained derivatives with electron-withdrawing groups (EWGs) (Figure-1). These compounds were obtained using a conventional method involving a Baker-Venkataramann rearrangement [26,27] and the general synthetic routes that were adapted for access to these flavones have been previously reported by our laboratory [28]. The purities of these compounds were established by reverse phase HPLC and the purities were found to be >95% in all cases.

The synthesised compounds (**1c-f** to **19c-f**) were evaluated for their ability to protect neurons from H_2O_2-induced oxidative stress in an *in vitro* model using the SH-SY5Y human neuroblastoma cell line [29,30]. First, an appropriate concentration of H_2O_2 for inducing oxidative stress in SH-SY5Y cells was determined by exposing the SH-SY5Y cells to various concentrations of H_2O_2 (5-500 µM) for 18 h. From the cell viability assessment, using the MTT assay, it was found that H_2O_2 at 130 µM (IC_{50} value) induces a 50% reduction in the SH-SY5Y cell viability (Supplementary info, Figure-S1). Hence, this concentration (130 µM) was used for further experiments to induce oxidative stress in SH-SY5Y cells.

In order to determine the protective effects of compounds **1c-f** to **19c-f** on H_2O_2-oxidative cell injury, SH-SY5Y cells were treated with each of these compounds at the physiologically relevant concentration of 3 µM concentration [31] for 24 h prior to exposure to H_2O_2 (18 h). The cell viability was determined by the MTT assay and the results are shown in Figure-2. Treatment with H_2O_2 at 130 µM exhibited an approximate 50% reduction of the cell viability in comparison to the control.

Effect of number of hydroxyls present on the flavonoid scaffold: Among series-1, comprising of flavones with differing number of hydroxyls, the hydroxy flavones **1d**, **1f**, **4d**, **4f** and **5d** (cell

viability**>**70%) showed significant protection of SH-SY5Y cells against H_2O_2 induced oxidative stress. Among the hydroxy flavones it was found that those bearing catecholic hydroxyl (*o*-hydroxy) substitutions either on ring-A or -B exerted significant neuroprotective effects, with the exception of **3d** and **3f.** In contrast, flavones that lack these functionalities (for example **2d** and **2f** with C-5,7 hydroxyl (*m*-hydroxy) groups) were found to be inactive (cell viability **<** 50%). This observation indicates that the presence of catecholic hydroxyl groups is necessary for the neuroprotective activity and this observation is in line with previous studies of natural flavones [32]. Comparison of the activities of **4d** and **5d** indicates that the presence of the C-3 hydroxyl group decreases the neuroprotective ability of flavonoids, however this observation is contradictory to a previous report that suggests that the hydroxy substitutions in the A-ring (C-5,7) and at position C-3 (C-ring) of the flavones are necessary to afford neuronal cell protection [33]. Also, no definite correlations were observed between the number of hydroxyls and neuroprotective ability. Among these derivatives (Series-1), the hydroxy 4-thioflavones were found to be less active than their corresponding hydroxy flavones. Further, the methoxy flavones (both 4-C=O and 4-C=S) were found to be inactive, which indicates that the presence of free hydroxyl groups is necessary for neuroprotective activity. In this series, compounds **1d** and **4d** (cell viability **=** 96.15 ± 4.02% and 98.37 ± 3.81%, for **1d** and **4d** respectively) were found to confer greater neuronal protection and were identified as the most active compounds.

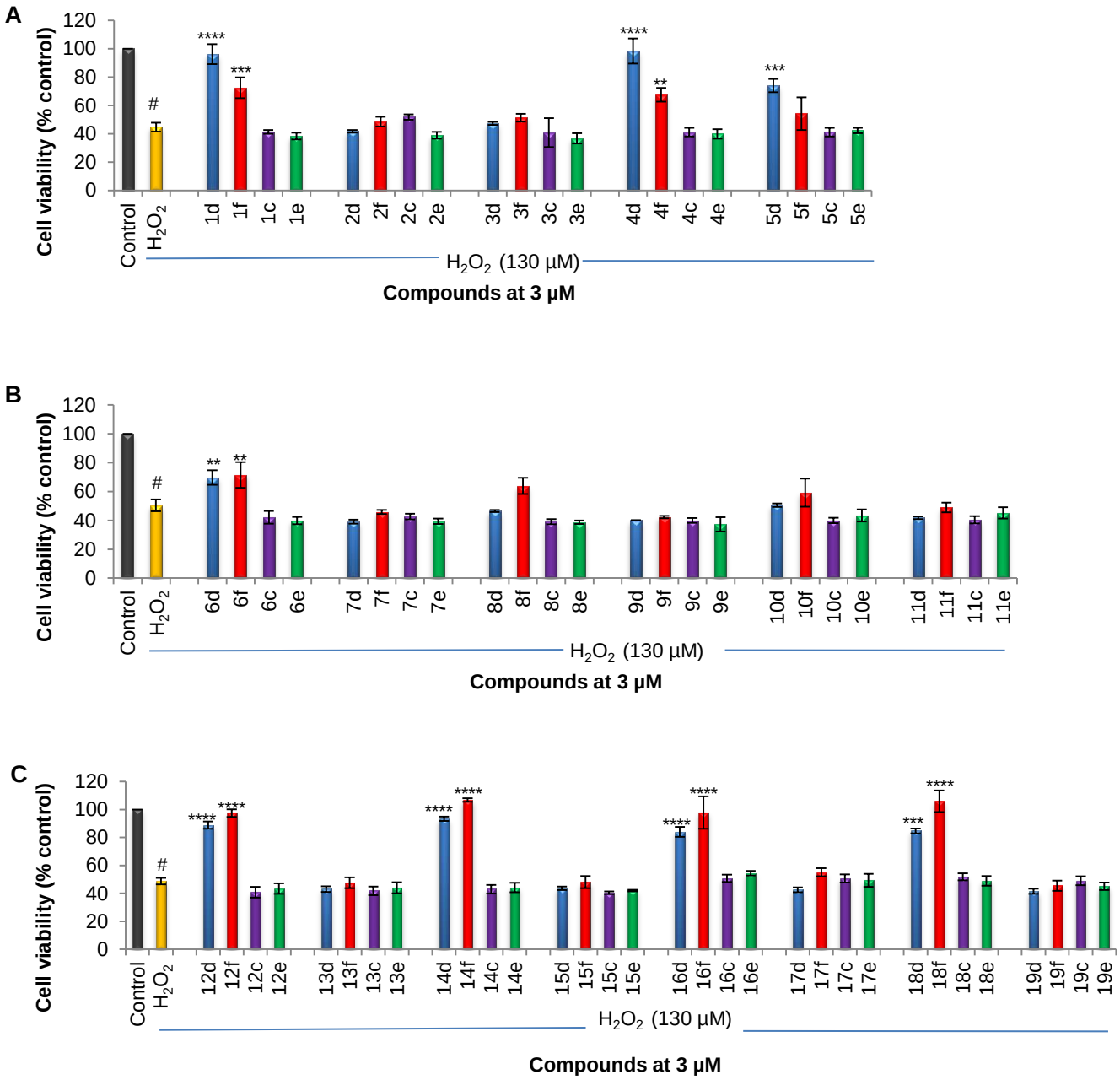


Figure 2. Neuroprotective effects of (A) compounds **1c-f to 5c-f** (B) compounds **6c-f to 11c-f** and (C) compounds **12c-f to 19c-f** at 3 µM concentration against H_2O_2-induced oxidative stress in SH-SY5Y cells. Cells without treatment serve as control. Cell viability was measured by MTT assay. Statistical significance was estimated by one-way ANOVA followed by Bonferroni's post hoc test, (#)-significance with respect to the control ($p < 0.0001$) and (*)-significance with respect to H_2O_2

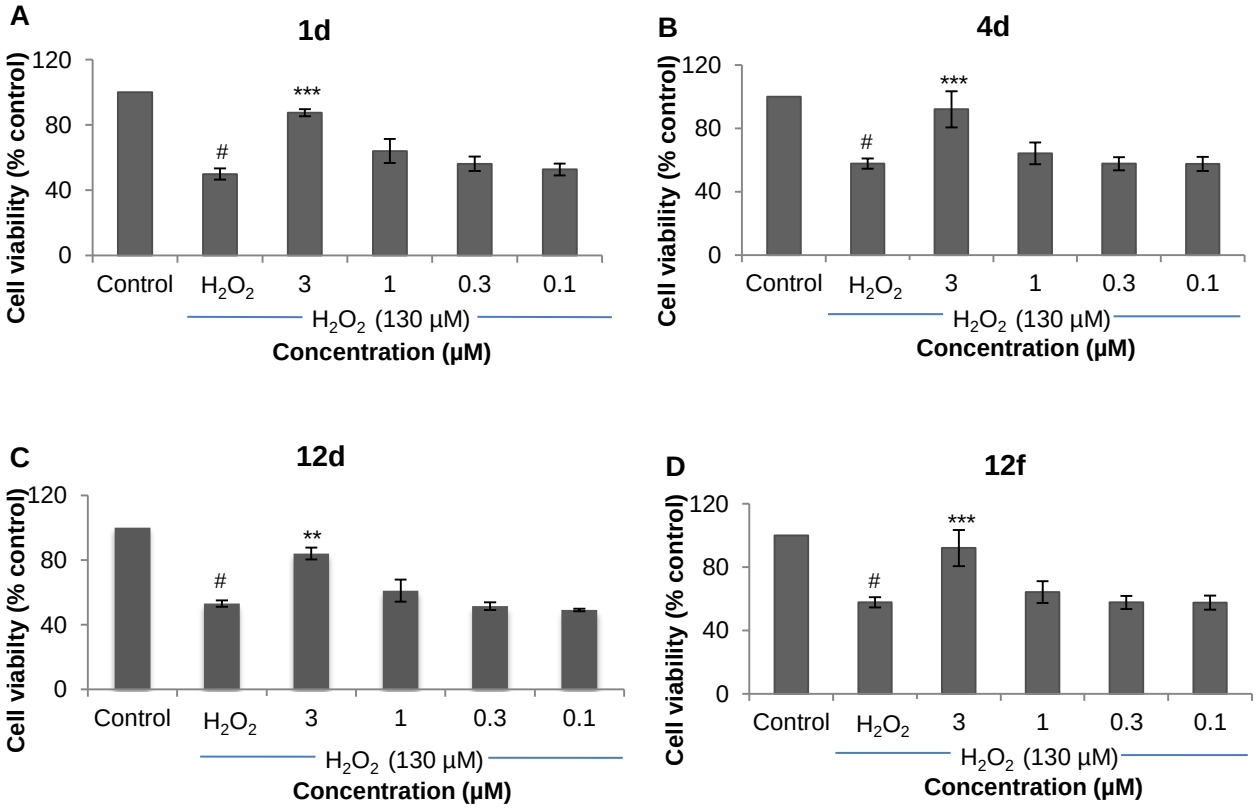
(**p < 0.01, ***p < 0.001 and ****p < 0.0001). Colour coding: blue-hydroxy flavone (-OH, 4-C=O), red-hydroxy 4-thioflavone (-OH, 4-C=S), purple-methoxy flavone (-OMe, 4-C=O) and green-methoxy 4-thioflavone (-OMe, 4-C=S).

Effect of bioisosteric replacement: To further explore the structure-activity relationships, the series of synthesised bioisosteric analogues (compounds **6c-f** to **11c-f**) were screened at 3 µM concentration for their neuroprotective properties. As shown in Figure-2A, replacement of the B-ring phenyl group in compound-**1d** with a 2-thienyl moiety resulted in a decreased neuroprotective activity (cell viability **=** 96.15 ± 4.02% vs 69.73 ± 4.81%, for **1d** and **6d** respectively), whereas replacement with the 2-furanyl and 3-pyridyl groups has resulted in a significant loss of neuroprotective activity (cell viability **<** 60%). As previously observed, the 5,7-regioisomeric analogues (both 4-C=O and 4-C=S) and the methoxy analogues (both 4-C=O and 4-C=S as well as 5,7-regiomers) were found to be inactive (cell viability **<** 50%). These observations therefore suggest that bioisosteric replacement of the B-ring phenyl group is detrimental for the neuroprotective activities.

Influence of electron-withdrawing groups: As part of the optimisation and systematic SAR evaluation, compounds **12c-f** to **19c-f** with electron-withdrawing substituents at the C-4' position of the B-ring phenyl moiety were analysed for their neuroprotective activities at 3 µM concentrations. The 7,8-hydroxy flavone and 4-thio flavone analogues **12d** (cell viability **=** 88.81 ± 2.53%), **12f** (cell viability **=** 97.40 ± 4.30%), **14d** (cell viability **=** 93.36 ± 1.47%), **14f** (cell viability **=** 106.75 ± 1.22%), **16d** (cell viability **=** 84.00 ± 3.60%), **16f** (cell viability **=** 97.85 ± 2.58%), **18d** (cell viability **=** 84.64 ± 1.69%), and **18f** (cell viability **=** 105.85 ± 3.72%) were found to be neuroprotective, whereas the 5,7-hydroxy and methoxy analogues exhibited poor neuroprotective activities (cell viability **<** 50%) (Figure-2C). Incorporation of electron withdrawing groups significantly enhanced the neuroprotective abilities of 7,8-dihydroxy thioflavones.

Overall, among the three series of flavone analogues, compounds **1d**, **4d**, **12d**, **12f**, **14d**, **14f**, **16d**, **16f**, **18d** and **18f** exhibited the highest neuroprotective effects at a single dose of 3 µM. Hence, these compounds were further evaluated at lower concentrations to determine their ability to exert neuronal protection against H_2O_2-induced oxidative stress. As shown in Figure-3, compounds **1d**, **4d**, **12d**, **12f**, **14d**, **16d**, **16f** and **18d** exerted neuroprotective effects only at 3 µM and no significant protective effects were observed at lower concentrations. Interestingly, the novel compounds **14f** and **18f** were found to be effective even at 0.3 µM concentration (cell viability **>**70%).

Further, the toxicity of compounds **1d**, **4d**, **12d**, **12f**, **14d**, **16d**, **16f** and **18d** was also determined by exposing the SH-SY5Y cells to 3 µM and 10 µM doses of these compounds for 24 h. The cell viability assessment (using the MTT assay) revealed that these compounds were not toxic to SH-SY5Y cells at the concentrations tested except for compound **16f** (52% reduction in the cell viability at 10 µM, $p < 0.001$) (Figure-4).



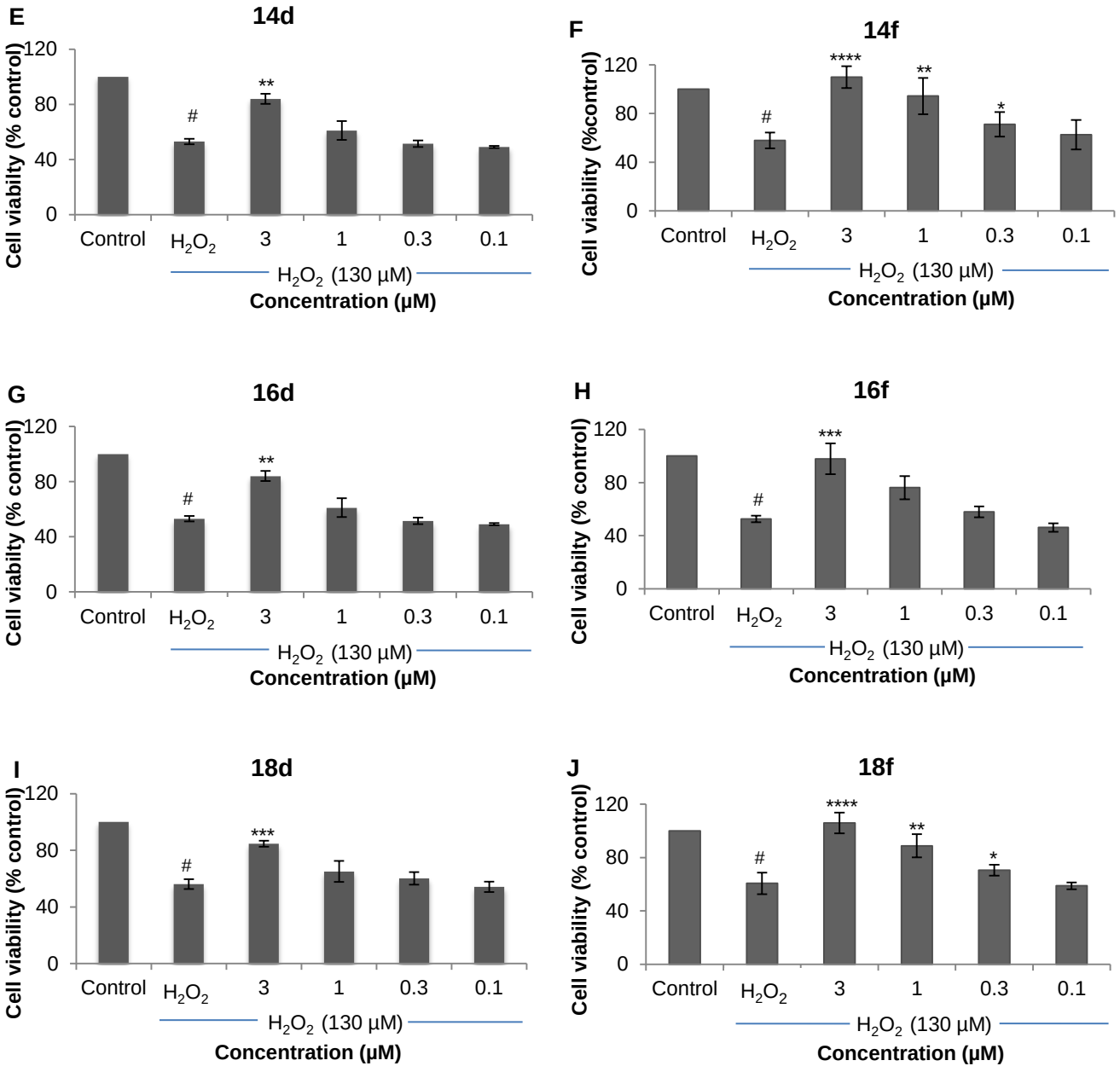


Figure 3. Neuroprotective effects of (A) Compound-**1d**, (B) Compound-**4d**, (C) Compound-**12d**, (D) Compound-**12f**, (E) Compound-**14d**, (F) Compound-**14f**, (G) Compound-**16d**, (H) Compound-**16f**, (I) Compound-**18d**, and (J) Compound-**18f** in a dose dependent manner in the concentration range 0.1-3 µM against H_2O_2-induced oxidative stress in SH-SY5Y cells. Cells without treatment serve as control. Data are expressed as the mean ± standard error of the mean (SEM) (n = 3). Statistical significance was estimated by one-way ANOVA followed by Bonferroni's post hoc test, (#)-significance with respect to the control ($p < 0.0001$) and (*)-significance with respect to H_2O_2 (**$p < 0.01$and *** $p < 0.001$).

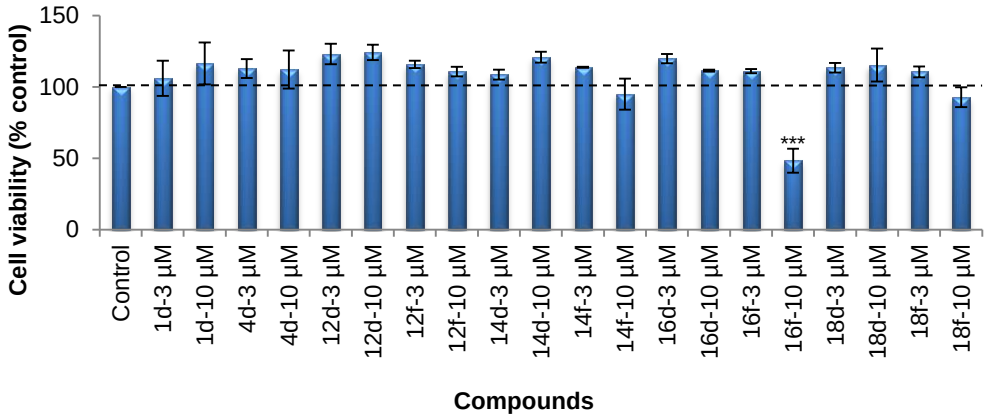


Figure 4. Toxic effects of compounds **1d**, **4d**, **12d**, **12f**, **14d**, **14f**, **16d**, **16f**, **18d** and **18f** evaluated at 3 and 10 µM concentrations against SH-SY5Y cells. Cells without treatment serve as control. Data are expressed as the mean ± standard error of the mean (SEM) (n = 3). Statistical significance was estimated with respect to the control by one-way ANOVA, followed by Bonferroni's post hoc test (***$p < 0.001$).

Taken together, the neuroprotective evaluation of these compounds (**1c-f** to **19c-f**) indicates that both the number and positions of the hydroxyl groups affects the neuroprotective abilities of flavones. Our results, in line with previous data [34], also highlight that the catecholic hydroxy substitution is indispensable for the neuroprotective activity. Systematic comparison of hydroxy flavones and methoxy flavones has also shown that the complete methylation of hydroxy groups renders the flavones inactive, hence, free hydroxyls are considered crucial for the neuroprotective activity. Therefore, one significant, novel contribution of this study is the demonstration that the B-ring phenyl group is important for neuroprotective activity. Moreover, a significant loss of neuroprotective activity of flavones, as observed with the bioisosteric replacement of the B-ring phenyl group, strongly suggests that π interactions of this phenyl ring play a key role in the neuroprotective activity. Also, in this study, the differential effects of 7,8-dihydroxy flavones (with 4-C=O) and 7,8-dihydroxy 4-thioflavones (with 4-C=S) and the substitution of EWGs have been shown. The correlation between the increased neuroprotective activity, and the presence of 4-C=S EWGs, suggests that replacement of 4-

C=O with 4-C=S and the substitution of EWGs are beneficial for neuroprotection, which is most likely to be a consequence of the increased hydrophobicity [34,35].

Molecular mechanism of action: Flavones can act either as a free radical scavenger (antioxidant) or by triggering intracellular pathways for cell survival. Therefore, to gain insight into the molecular mechanism of action underlying the neuroprotective activities of these flavones, their antioxidant potentials and impact on certain intracellular signalling targets were explored.

Antioxidant activity: Flavones are very well known for their antioxidant activities [2,36], therefore the antioxidant properties of flavones **1d** (7,8-dihydroxy flavone), **4d** (luteolin, a well-known natural flavone) and compound-**14d** with a 4-C=O moiety, as well as the neuroprotective 4-thioflavones (with 4-C=S) **14f**, **16f** and **18f** were studied. For this, the flavones were evaluated at their neuroprotective concentration of 3 μM both for their ability to directly interact with free radicals (primary antioxidant activity) using a DPPH free radical scavenging assay and for their ability to bind to ferrous (Fe^{2+}) ion that catalyses oxidation (secondary antioxidant activity), using a metal chelating assay (Table-1). As shown in Table 1, all the aforementioned flavones showed very low scavenging activity (only up to 1.9% inhibition of DPPH radical) at 3 μM, at which neuroprotection by these flavones was observed. Also, based on previous reports on DPPH scavenging data for the well-known flavones (**1d** and **4d**, Series-1), no correlation was found between the order of their neuroprotective abilities [**1d** (Cell viability-97%) > **4d** (Cell viability-73%, $p < 0.05$)] and the order of their scavenging activity [DPPH radical scavenging activity- IC_{50}-**4d** (11.04 ± 0.38 μM) > **1d** (15.50 ± 0.12 μM)] [37]. Since, in general, compounds with catechol groups are defined as effective metal chelators [38–40], the iron-chelating ability of flavones **1d**, **4d**, **14d**, **14f**, **16f** and **18f** at 3 μM was further studied. Interestingly, a low degree of Fe^{2+} chelation (only up to 5%) was exhibited by these flavones at 3 μM concentration (Table 1).

Taken together, it can be concluded that these flavones possess weaker antioxidant activity at their tested concentration, and hence antioxidant activity alone may not be sufficient to explain their observed neuroprotective effects. Therefore, to further probe the molecular mechanism of these flavones their affects on intracellular signalling pathways were explored.

Table 1. Antioxidant activities of flavones

Flavones	Antioxidant activity at 3 μM concentration[a]	
	DPPH scavenging assay (Scavenging activity, %)	Metal chelating assay (Fe^{2+} chelating activity, %)
1d	1.4 ± 0.2	3.7 ± 1.0
4d	1.9 ± 0.2	3.4 ± 0.4
14d	1.4 ± 0.5	3.7 ± 1.2
14f	1.7 ± 0.2	5.0 ± 1.6
16f	1.6 ± 0.6	4.4 ± 1.7
18f	1.3 ± 0.2	3.9 ± 0.4
Ascorbic acid[b]	1.3 ± 0.5	-
EDTA[c]	-	6.9 ± 0.4

[a] Data expressed as Mean ± SEM, n = 3; [b]Ascorbic acid was used as a reference standard for the DPPH scavenging assay, IC_{50} value of ascorbic acid for the inhibition of DPPH radical formation was established to be 330 ± 0.5 μM (Supplementary info, Figure-S2); [c]EDTA was used as a reference standard for the metal chelating assay, IC_{50} value of EDTA in metal chelating assay was determined to be 42 ± 0.8 μM (Supplementary info, Figure-S3).

Intracellular signalling: Accumulative evidence has shown that flavonoids display signalling properties during neuroprotection [17,41,42] [43] [44,45]. Hence, we explored the potential intracellular targets involved in the neuroprotective function of flavones. For this, the signal mediated in SH-SY5Y cells by the well-known compounds **1d**, **4d** (luteolin) and compound-**14d** with a 4-C=O moiety, as well as the neuroprotective 4-thioflavones (with 4-C=S) **14f**, **16f** and **18f,** were studied. Pooled samples from three independent treatments of SH-SY5Y cells with and without H_2O_2 (130 μM), and with these compounds at 3 μM concentration for 24 h followed by the exposure to H_2O_2 (1 h) [42], were analysed using the PathScan® Intracellular Signalling Array Kit (Figure-5). These compounds were found to modulate the signalling molecules that are associated with cellular survival and apoptosis such as ERK1/2, mTOR, AMPKα and Akt targets such as Bad and PRAS40. Treatment of SH-SY5Y cells with H_2O_2 (130 μM)

resulted in a marked reduction in phosphorylation of ERK1/2, mTOR, Bad and PRAS40. Pre-treatment with 7,8-dihydroxy flavones (containing 4-C=O) showed that compounds **1d** and **4d** were able to confer neuroprotection by the inhibition of apoptosis through restoration of Bad (a pro-apoptotic protein) phosphorylation (by inactivating its apoptotic activity), whereas, compound **14d** was shown to elicit its activity through restoration of mTOR phosphorylation, which restores protein synthesis. In the case of 4-thioflavones, compound **14f** significantly restored phosphorylation of ERK1/2, mTOR, Bad and PRAS40 up to the same levels or higher than that observed in the control, along with activation of AMPKα. Also, compound **18f** was found to restore phosphorylation of ERK1/2 and mTOR, along with activation of AMPKα, however, compound **16f** showed restoration of Bad phosphorylation only. These results support the *in vitro* observations and suggest that the neuroprotective effects of flavones are mediated via ERK1/2 and PI3K/Akt/mTOR pathways and that these flavones differentially activate the pro-survival protein kinases based on their chemical structure. Also, a comparison of intracellular signalling of the 4-thioflavone **14f** with its corresponding flavone **14d** highlights the positive influence of the 4-C=S substitution on the neuroprotective activity. Indeed, the enhanced neuroprotective effects of compounds **14f** and **18f** can be attributed to their potential to modulate multiple signalling targets than their antioxidant activity.

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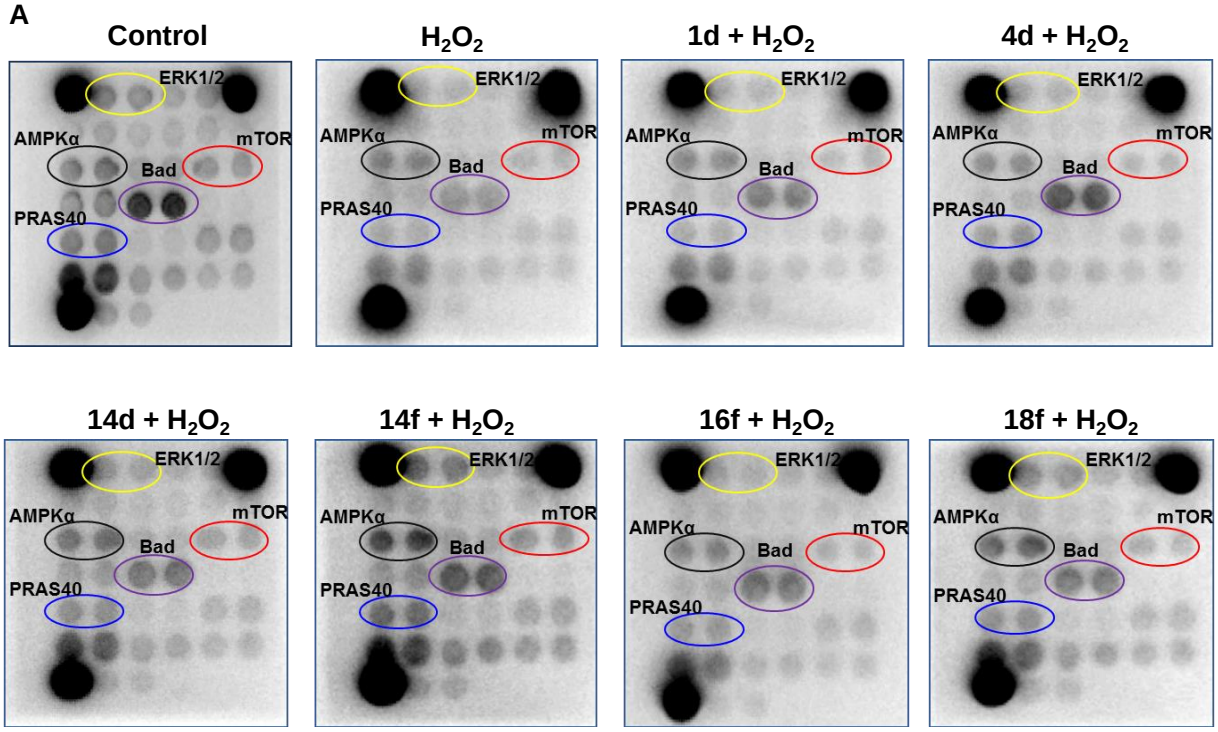
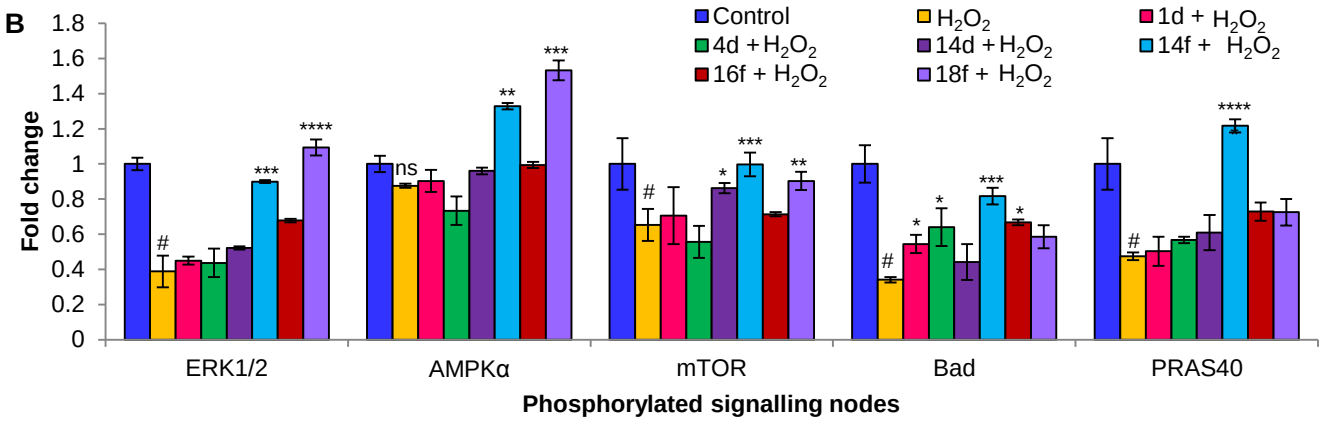

Control
H2O2
1d + H2O2
4d + H2O2
14d + H2O2
14f + H2O2
16f + H2O2
18f + H2O2
ERK1/2
AMPKα
mTOR
Bad
PRAS40



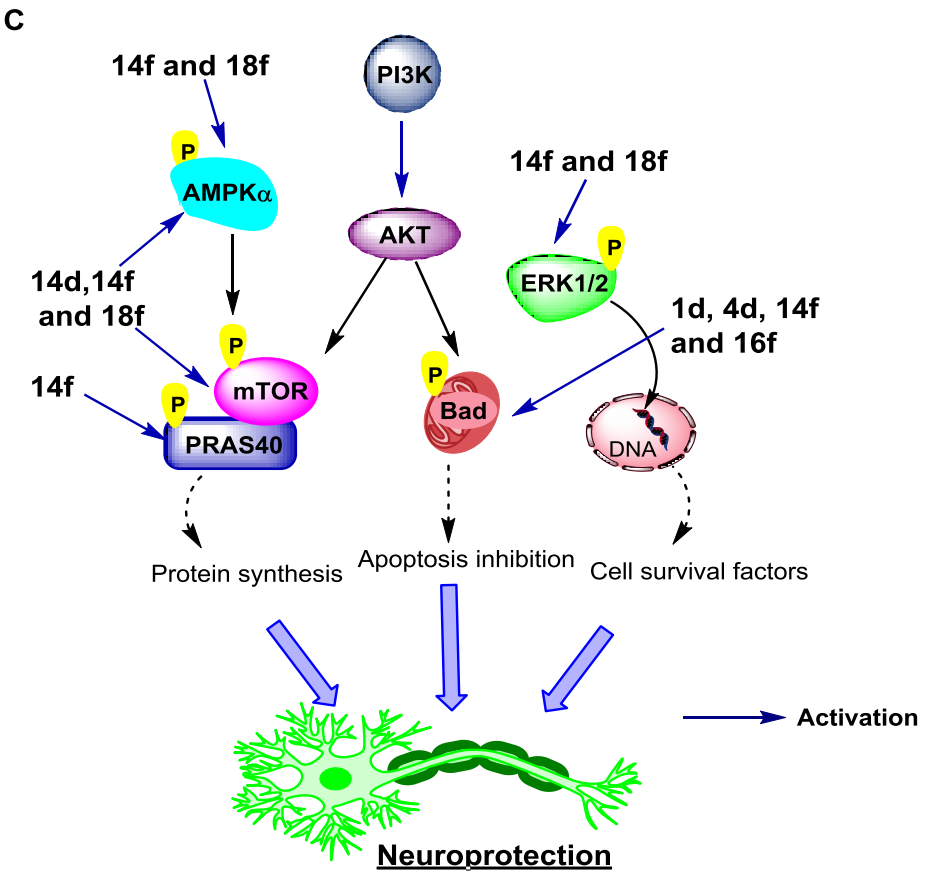


Figure 5. (A) Chemiluminescent array images of the PathScan Intracellular Signalling array kit revealing various phosphorylated in SH-SY5Ycells pre-treated with and without compounds **1d**, **4d**, **14d**, **14f**, **16f** and **18f** at 3 µM for 24 h before exposure to H_2O_2 (130 µM). Cells without treatment serve as control. (B) Bar chart representing the fold change in

the integrated density of phosphorylated signalling nodes in the array image of SH-SY5Ycells pre-treated with and without compounds **1d**, **4d**, **14d**, **14f**, **16f** and **18f** at 3 μM for 24h before exposure to H_2O_2 (130 μM). Data are expressed as the mean ± standard error of the mean (SEM) (n = 4). Statistical significance was estimated with respect to the control (#) and with respect to H_2O_2 (*) by one-way ANOVA, followed by Bonferroni's post hoc test (ns-not significant, *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.001). (C) Molecular mechanism of action of **1d**, **4d**, **14d**, **14f**, **16f** and **18f** in SH-SY5Y cell line.

Correlation between neuroprotective activity and BBB permeability : This correlation was probed by plotting the neuroprotective ability of each of the flavones against its predicted BBB score (Figure-6). This showed that the novel compounds **14f** and **18f** possess both high neuroprotective ability (cell viability > 95%, neuroprotective even at 0.3 μM concentration) and high BBB permeability (BBB score > 0.02). In contrast, the natural/well-known flavones such as 7,8-dihydroxy flavone (**1d**), luteolin (**4d**) and quercetin (**5d**) were found to be neuroprotective with poor BBB permeability. Therefore, the novel thioflavones **14f** and **18f** can be considered as lead candidates for further design and development of neuroprotective agents.

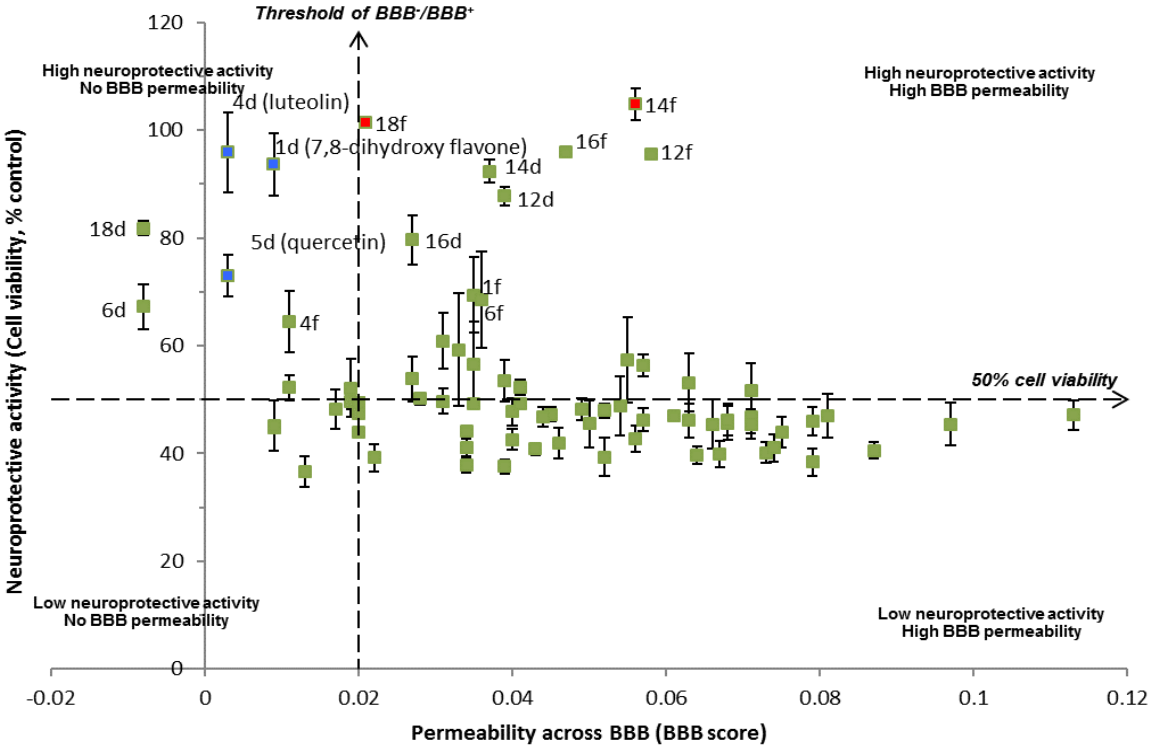


Figure 6. Graph represents BBB permeability of compounds (predicted BBB score) versus neuroprotective activity (cell viability) determined at 3µm using the SH-SY5Y neuroblastoma cell line. The dashed line at x = 0.02 represents the threshold BBB-/BBB+ score and the dashed line at y = 50 represents 50% cell viability in SH-SY5Y cells exposed to H_2O_2. Neuroprotective natural flavone/well-known flavones are highlighted in blue and the thioflavones that have been identified to be neuroprotective are highlighted in red. Lower left quadrant represents low neuroprotective activity and no BBB permeability, lower right quadrant represents low neuroprotective activity and high BBB permeability, upper left quadrant represents high neuroprotective activity and no BBB permeability and upper right quadrant represents high neuroprotective activity and high BBB permeability.

CONCLUSIONS

In summary, evaluation of the neuroprotective properties of structurally related methoxy flavones, hydroxy flavones and their 4-thio analogues has provided significant insights into the SARs of flavones, as summarised in Figure 7.

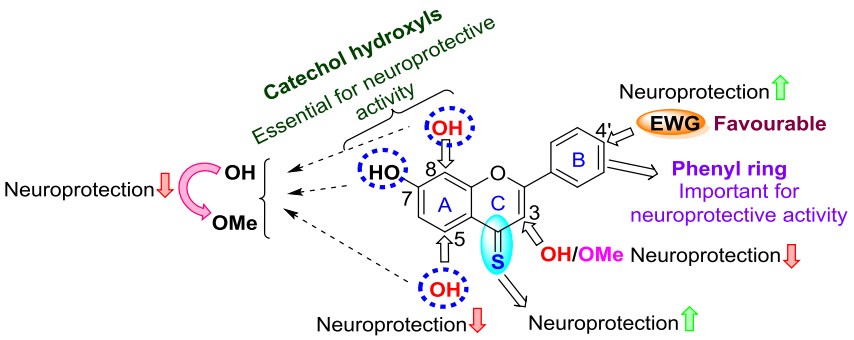


Figure 7. Summary of the SARs on the neuroprotective activities of flavones.

In particular, the presence of catecholic hydroxyl groups on ring-A, and the presence of the B-ring phenyl group, were found to be essential for neuroprotection. In addition, the presence of EWGs at C4' of the B-ring phenyl, as well as 4-C=S substitution, were found to be beneficial for neuroprotection. In general, 7,8-dihydroxy 4-thioflavones such as compounds **14f** and **18f** were found to exhibit potent neuroprotective effects against H_2O_2-induced oxidative stress with their activity being restored even at 0.3 µM concentration. Investigation of the molecular mechanism of action of key leads indicated that these compounds preferably mediate their neuroprotective effects through suppression of apoptosis by activating the anti-apoptotic proteins, and inactivating the pro-apoptotic proteins of the ERK1/2 and PI3K/Akt/mTOR pathways. Overall, the neuroprotective compounds identified in this study provide promising leads for further optimisation and development of potent flavone based neuroprotective agents.

FUTURE PERSPECTIVE

Increased levels of oxidative stress are closed linked with many neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, and Amyotrophic Lateral Sclerosis [46–48]. Hence, therapeutic strategies aimed at identifying small molecules that are capable of conferring

neuroprotection against oxidative stress are of significant importance for developing effective treatment for these neurodegenerative diseases [14,49]. In this context, identification of novel neuroprotective agents with favourable pharmacokinetic profiles and CNS distribution is of pivotal importance. Therefore, the novel flavones **14f** and **18f,** with potential neuroprotective activities, along with favourable BBB permeability, can be considered as promising candidates for further optimisation and development as neuroprotective agents. In this regard, future studies that will decipher the pharmacokinetic and pharmacodynamic properties of these novel compounds will further guide the optimisation of these candidates for neuroprotective applications.

EXCECUTIVE SUMMARY

• A library of 76 structurally related flavones have been screened for their neuroprotective activities against H_2O_2-induced oxidative stress.

• Substitution of 4-C=S and EWGs at C4' of the B-ring phenyl resulted in compounds with enhanced neuroprotective properties.

• Further structural optimisation of the identified novel lead candidates, and *in vivo* evaluation, are required to develop novel neuroprotective agents.

ASSOCIATED CONTENT

Supporting Information Available: This includes the dose-dependent curves of H_2O_2, ascorbic acid and EDTA. Theoretical BBB scores calculated using the online prediction tools are also given.

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ABBREVIATIONS

MTT-3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, DPPH-2,2-diphenyl-1-picrylhydrazyl, EDTA-Ethylenediaminetetraacetic acid, ERK1/2-extracellular signal-regulated kinase, mTOR-mammalian target of rapamycin, AMPKα-5' adenosine monophosphate-activated protein kinase, GSK-3β- Glycogen synthase kinase-3β, Bad-Bcl-2-associated death promoter, PRAS40-proline-rich Akt substrate of 40 kDa, PI3K-Phosphatidylinositol-4,5-bisphosphate-3-kinase.

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